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A novel prohormone processing site in *Aplysia californica*: the Leu–Leu rule

Amanda B. Hummon, He-Qing Huang,[1] Wayne P. Kelley and Jonathan V. Sweedler

Department of Chemistry and the Beckman Institute, University of Illinois, Urbana, Illinois, USA

Abstract

Neuropeptides are a complex set of signaling molecules produced through enzymatic cleavages from longer prohormone sequences. The most common cleavage sites in prohormones are basic amino acid residues; however, processing is observed at non-basic sites. Cleavage at Leu–Leu sequences has been observed in three *Aplysia californica* prohormones. To further investigate this unusual event, native and non-native synthetic peptides containing Leu–Leu residues are incubated with homogenates of *Aplysia californica* ganglia and the resulting products monitored with MALDI MS. Cleavage near and between Leu–Leu residues is observed in the abdominal and buccal ganglia homogenates, confirming the presence of an unidentified peptidase. In addition, fractions from an HPLC separation of buccal ganglia homogenates also produce cleavages at Leu–Leu residues. Products resulting from cleavage at Leu–Leu sites are observed and are produced in larger amounts in acidic and neutral pH ranges, and cleavage is inhibited by the addition of EDTA, suggesting a metal is required for activity.

Keywords: leucine–leucine cleavages, MALDI MS, neuropeptides, prohormone processing.

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The remarkable chemical complexity of the brain renders studying the production of intercellular signaling molecules a challenge. The neuropeptides are produced and modified to form a myriad of products following cleavage from larger precursor prohormone structures. A working knowledge of the most frequently used cleavage sites aids in the prediction of new neuropeptides from novel prohormone genes. In the vernacular of prohormone processing, there are certain residues, namely basic amino acid residues that are recognized as cleavage sites. While the most ubiquitous sites for cleavage are these basic residues, there are also some additional amino acid combinations that can be used as cleavage points in the prohormone. We report one such unexpected cleavage site, Leu–Leu residues, observed as cleavage loci in three *Aplysia californica* prohormones.

The processing of neuropeptides is controlled by a large number of enzymatic processing steps. An unusual cleavage site suggests a novel endoprotease, in this case, perhaps one that is responsive to hydrophobic amino acid residues. This study seeks to confirm the processing of peptides and prohormones at Leu–Leu sites and to present some preliminary investigations of the nature of the endoprotease responsible. In order to determine the cleavage point in the Leu–Leu peptides, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) was used. MALDI MS is an excellent method to probe the presence and production of neuropeptides in neuronal tissue (van Veelan *et al*. 1993; Jimenez *et al*. 1998; Li *et al*. 2000). In this study, peptides of known molecular weight containing Leu–Leu sites were added to cellular homogenates and then monitored with MALDI MS to fully characterize the Leu–Leu cleavages.

Cleavage at Leu–Leu sites, catalyzed by the enzyme renin, occurs in numerous species. Renin is a highly substrate-specific enzyme, responsible for the conversion of the glycoprotein, renin substrate, into angiotensin I by cleavage between the two Leu residues. Angiotensin I is then further converted by the angiotensin converting enzyme to produce angiotensin II which affects the contraction of smooth muscle and is one of the most powerful pressor substances known

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Address correspondence and reprint requests to Jonathan V. Sweedler, Department of Chemistry and the Beckman Institute, University of Illinois, Urbana, IL 61801, USA. E-mail: sweedler@scs.uiuc.edu

[1]The present address of He-Qing Huang is the Department of Biology and The Center of Analysis and Testing, Xiamen University, China.

Abbreviations used: AP, acidic peptide; ASW, artificial sea water; DHB, 2,5-dihydroxybenzoic acid; ELH, egg-laying hormone; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry; RP-HPLC, reversed phase high-performance liquid chromatography.

(Skeggs *et al*. 1980). Renin has been isolated in many vertebrates, but, thus far, an invertebrate analog has only been identified in a single species, the leech, *Theromyzon tessulatum* (Laurent and Salzet 1995; Salzet and Stefano 1997). To our knowledge, renin has not been isolated in *Aplysia californica*. However, angiotensinogen-like immunoreactivity was demonstrated in portions of the *Aplysia* CNS, primarily in the F-cluster of the cerebral ganglion (Gonzalez *et al*. 1995). The Leu–Leu selectivity that we report, however, differs significantly from known renin enzymes. Another enzyme known to cleave at leucine residues is chymotrypsin. Chymotrypsin cleaves preferentially at the acyl bonds of tyrosine and phenylalanine, and at a much slower rate at Leu residues (Bauer *et al*. 1976). Hydrolysis by chymotrypsin occurs selectively on the C-terminus side of the targeted residue. As the specificity of the observed cleavage is significantly different, we verify the Leu–Leu cleavage and characterize the activity of this peptidase.

Leu–Leu cleavage has previously been observed in *Aplysia* egg-laying hormone (ELH)-related prohormones (Nagle *et al*. 1988; Garden *et al*. 1998). To further characterize Leu–Leu processing, native and non-native synthetic peptides were prepared containing Leu–Leu sites. These peptides were incubated with homogenates of *Aplysia* ganglia and their processing monitored by MALDI MS. Leu–Leu processing was expected in the abdominal, cerebral, and pedal ganglia based on prior work with prohormones containing Leu–Leu sites. Abdominal ganglia were studied as a portion of the CNS where this processing was expected. In contrast, the buccal ganglia were studied as a portion of the CNS where this processing was *not* known to occur to determine how widespread such processing is within the CNS. Additional homogenate samples were separated by RP-HPLC into fractions, incubated with Leu–Leu containing peptides, and monitored by MALDI MS. We report here that such processing is widespread throughout the *Aplysia* CNS, and we observe unique ganglia-specific Leu–Leu processing for all three synthetic peptides, indicating this step is a tightly regulated event in *Aplysia* prohormone processing.

Experimental procedures

Animals

Aplysia californica weighing 10–200 g were obtained from the Aplysia Research Facility (Miami, FL, USA). Larger animals were collected off the Monterey Peninsula, CA, USA. Animals were maintained in artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH, USA) at 14°C.

Chemicals

Acetone and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). MALDI matrices used were 2,5-dihydroxybenzoic acid (DHB) (ICN Biomedical, Aurora, OH, USA) and α-cyano-4-hydroxy-cinnamic acid (CHCA) (Aldrich, Milwaukee, WI, USA).

Trifluoroacetic acid (TFA), human secretin (HSDGTFTSELSRLREGARLQRLLQGLVa, 3040.4 Da) and porcine angiotensinogen 1–14 (DRVYIHPFHLLVYS, 1758.9 Da) were obtained from Sigma (St Louis, MO, USA) as were the chemicals used to prepare artificial sea water (ASW). *Aplysia* acidic peptide (SSGVSLLTSNKDEEQRELLKAISNLLD, 2961.3 Da) was synthesized by the University of Illinois Biotechnology Center (Urbana, IL, USA). Aqueous solutions were prepared with purified water (Millipore, Bedford, MA, USA). EDTA, Trizma Acid and Trizma Base were obtained from Sigma (St. Louis, MO, USA).

Leu–Leu homogenate incubations

Abdominal and buccal ganglia were removed from 12 *Aplysia californica* (100–200 g) in the presence of 14°C ASW and transferred to 500 μL polypropylene tubes. A volume of ASW (40 μL) was added to each ganglion prior to homogenization. The ganglia were homogenized first using tungsten needles followed by 20 s of sonication. After homogenization, the ganglia were centrifuged for 2 min at 14 400 *g*. The resulting supernatant was separated into two ~20 μL portions in 500 μL polypropylene tubes. Additional sample tubes contained ASW as controls. One set of samples (homogenate supernatant, ASW) was heated for 10 min at 95°C. One microliter volumes were sampled from each of the four vials prior to the addition of the Leu–Leu peptide solution. These samples were mixed with 3 μL of fresh DHB matrix solution. After initial sampling, 0.5–10 μL of a Leu–Leu peptide solution was added to each of the four vials and periodic sampling for mass spectrometry (MS) performed. The concentrations and volumes of the Leu–Leu peptide solutions used were as follows: *Aplysia* acidic peptide, 3 mM, 0.5–10 μL; porcine angiotensinogen, 0.74 mM, 2 μL; human secretin, 1 mM, 5 μL. The incubation of acidic peptide in the buccal ganglia was repeated multiple times with different volumes of peptide solution. All experiments were performed for 15 min following addition of the peptide solution. Spectra were also obtained of ganglia homogenates prior to peptide incubation.

Microbore RP-HPLC separation of buccal ganglia homogenates

Buccal ganglia were pooled from 15 *Aplysia californica* (> 500 g) for RP-HPLC separation. Samples were homogenized in a microhomogenizer (Jencons Scientific Ltd, Bedfordshire, UK), sonicated (model 2200; Branson, Danbury, CT, USA), and were centrifuged (Biofuge 15; Baxter, McGraw Park, IL, USA). A microbore HPLC instrument (Magic 2002; Michrom BioResources, Auburn, CA, USA) was used in the separation. Twenty microliters of the extract were injected onto a 0.5 × 150 mm Reliasil column (Michrom Bioresources), consisting of a 5 μm particle size and a 30-nm pore size C-18 packing. The column was equilibrated with Solvent A at a programmed temperature of 35°C, and a gradient was developed from 5 to 98% of solvent B in 34 min. Solvent A consisted of 2% acetonitrile/~98% H_2O + 0.1% formic acid and 0.02% TFA and solvent B being 95% acetonitrile/~5% H_2O + 0.1% formic acid and 0.02% TFA. Sample peaks were detected via absorbance at 214 and 280 nm wavelengths and the eluant collected by a small volume fraction collection system (Gilson FC 203B, Middletown, WI, USA). To identify the peptides of interest, each fraction was screened using MALDI MS; approximately 0.50 μL of each fraction

was deposited on a MALDI MS sample target followed by the same volume of a 2,5-dihydrobenzoic acid (10 mg/mL in H_2O). Thus more than 95% of each fraction was available for further assays.

RP-HPLC fraction screening

The buccal ganglia HPLC fractions were analyzed by MALDI MS. Eight microliters of each fraction was removed and combined with 2 μL of a Leu–Leu peptide solution (AP, $\sim 3 \times 10^{-5}$ M). The solution was incubated in a water bath (35°C) for 2 h. Following incubation, the fractions were analyzed by MALDI MS to identify the fraction containing the Leu–Leu endopeptidase. The active fractions were set aside for further testing.

pH study with RP-HPLC fractions

One of the RP-HPLC fractions previously identified to contain the Leu–Leu endopeptidase was used. Three 8 μL portions of the fraction were mixed with ~40 μL of a combination of two solutions, Solution A and Solution B. Solution A was prepared with 547 mg Trizma Base in 100 mL H_2O and had a pH of 9.3. Solution B was prepared with 702 mg Trizma Acid in 100 mL H_2O and had a pH of 4.9. The HPLC fraction aliquots were combined with these solutions to produce three samples of 5.5, 7.2 and 9.1 pH. The same time course experiment described in the Leu–Leu homogenate incubations section was performed with an 8 μL volume of $\sim 3 \times 10^{-5}$ M AP added. All experiments were performed for 15 min following addition of the peptide solution. Spectra were also obtained of ganglia homogenates prior to peptide incubation.

EDTA study with RP-HPLC fractions

One of the RP-HPLC fractions previously identified to contain the Leu–Leu endopeptidase was used. An 8 μL volume of the fraction was added to a 0.025 M Tris-HCl buffer solution at pH 7.25. A 20-mM solution of EDTA was prepared and 2 μL was added to the buffered solution. The mixture was heated at 35°C for 15 h. Roughly 2 μL of the solution was removed for MALDI analysis and the remainder of the solution was transferred to a specially constructed dialysis apparatus. In the apparatus, the sample was placed in a centrifuge tube with the tip removed and replaced with a membrane (< 3500 MW permeable). The centrifuge tube was suspended in a dialysis solution of Tris-HCl buffer. After 2 h, a 2 μL volume of the fraction solution was removed, reacted with 2 μL of AP (3×10^{-5} M), and analyzed by MALDI MS. To reactivate the endopeptidase, a 3–5 μL sample of the dialyzed fraction solution was mixed with 3 μL of tap water. After 3 h, a 2 μL volume of the reactivated fraction solution was removed, reacted with 2 μL of AP (3×10^{-5} M), and analyzed by MALDI MS.

Mass spectrometry

Mass spectra were obtained as previously described (Floyd *et al*. 1999), using a Voyager DE-STR mass spectrometer with delayed ion extraction (PE Biosystems, Framingham, MA, USA). A pulsed nitrogen laser (337 nm) was used as the desorption/ionization source. The instrument was used in linear mode, positive ion polarity with a 20-kV acceleration voltage. The mass spectra shown were unsmoothed and an average of 30–100 laser pulses. Mass calibration was performed externally on the same target by using a low mass and a high mass synthetic peptide, metenkephalin and bovine insulin, both purchased from American Peptide (Sunnyvale, CA, USA).

Results

Cleavage at Leu–Leu residues was observed in three *Aplysia* prohormones, the egg-laying hormone prohormone (Garden *et al*. 1998), the insulin prohormone (Floyd *et al*. 1999), and one of the myomodulin prohormones (Li *et al*. 1998). To further investigate processing at Leu–Leu sites, synthetic peptides were prepared containing Leu–Leu residues. The peptides were incubated with homogenates from specific *Aplysia* ganglia and monitored by MALDI MS. In all experiments, the supernatant of ganglia homogenates was used for the incubation. Also, the experiments were performed both at room temperature (22°C) and after incubation at 95°C, to verify that any observed Leu–Leu cleavages were enzymatic and not from nonenzymatic peptide degradation or a measurement artifact. No Leu–Leu processing was observed with samples heated to 95°C.

The MALDI matrix most commonly utilized with cellular samples is 2,5-dihydroxybenzoic acid (DHB). DHB has a pH of ~2, acidic enough to inhibit proteolytic activity (Garden *et al*. 1998) and, so that once the homogenate is introduced to the matrix, processing is stopped. Microliter samples of the homogenate/peptide mixture were removed at regular intervals and mixed with the MALDI matrix, thereby halting the enzymatic processing and preserving the relative amounts of the products for MALDI analysis.

The Leu–Leu processing varied widely for the three peptides and in the two ganglia (abdominal and buccal) homogenates studied. For *Aplysia* acidic peptide (AP), there was an obvious difference between the two ganglia (Fig. 1). In the buccal ganglia, AP is processed into multiple Leu–Leu products, appearing in a time-dependent manner. Larger Leu–Leu products are produced early in the run and then are cleaved to form smaller peptides (Fig. 2). Interestingly, almost all the observed products can be attributed to cleavage at Leu–Leu sites. Cleavage occurs with relatively equal percentages adjacent to the Leu–Leu sites (both at the N-terminus and the C-terminus), and between the two Leu–Leu residues, although the lack of quantitation in MALDI makes determining precise cleavage ratios problematic. Several of the AP processing products are from peptides cleaved between two pairs of Leu–Leu residues, thus Leu residues flank both ends of the peptide, resulting in three possible peptides of identical mass (e.g. TSNKDEEQRE**LL** and **L**TSNKDEEQRE**L** and **LL**TSNKDEEQRE). Therefore, without performing sequencing experiments, the observed masses may correspond to one or more of the specific peptide sequences. Given the low intensity of many of the products, post-source decay (PSD) sequencing techniques were not used to resolve them.

Processing of AP in the abdominal ganglia homogenates differed from the processing observed in the buccal ganglia homogenates. When preparing the abdominal ganglia for homogenization, the bag cell clusters were removed to

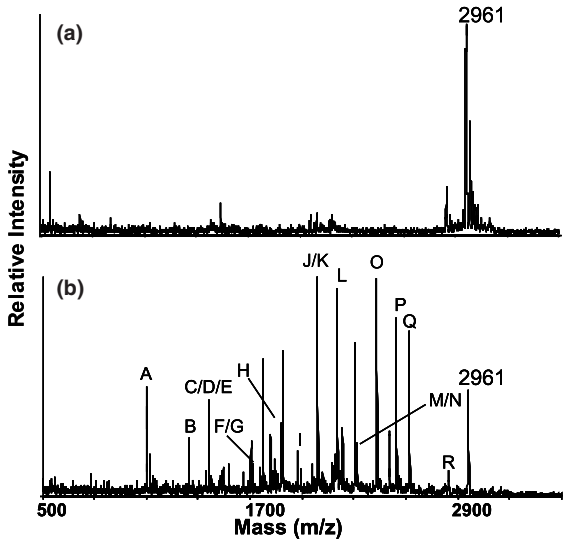

Fig. 1 Mass spectra of incubations of *Aplysia* acidic peptide (SSGVSLLTSNKDEEQRELLKAISNLLD, $[M + H]^+ = 2961$ Da) in the supernatant of abdominal (a) and buccal (b) ganglia homogenates at room temperature. No Leu–Leu processing products are observed in the abdominal ganglia homogenate, while numerous peptides resulting from Leu–Leu cleavage are produced in the buccal ganglia homogenate. The L-L peptide products observed in Fig. 1(b) are: A, **LL**KAISN**LL**D; B, TSNKDEEQRE**L**; C, **LL**TSNKDEEQRE; D, TSNKDEEQRE**LL**; E, **L**TSNKDEEQRE**L**; F, **L**TSNKDEEQRE**LL**; G, **L**TSNKDEEQRE**L**; H, SSGVS**LL**TSNKDEEQRE; I, TSNKDEEQRE**LL**KAISN; J, TSNKDEEQRELLKAISN**L**; K, **L**TSNKDEEQRELLKAISN; L, **L**TSNKDEEQRELLKAISN**L**; M, **LL**TSNKDEEQRE**LL** KAISN**L**; N, **L**TSNKDEEQRE**LL**KAISN**LL**; O, **L**TSNKDEEQRELLKAISN**LL**D; P, **LL**TSNKDEEQRELLKAISN**LL**D; Q, SSGVS**LL**TSNKDEEQRE**LL**KAISN; R, SSGVS**LL**TSNKDEEQRE**LL**KAISN**LL**.

reduce the native AP signal. AP is one of the peptide products of the egg-laying hormone (ELH) prohormone and is naturally produced in the bag cells of the abdominal ganglia. Not surprisingly, in the spectra acquired from some of the abdominal ganglia homogenates prior to peptide addition, AP and one of its processing products, AP S[1–20]K ($[M + H]^+ = 2234$ Da) were observed. Production of AP S[1–20]K would involve cleavage at a monobasic Lys residue and has often been observed in previous abdominal ganglia spectra acquired. However, no Leu–Leu processing products of AP were observed in the abdominal ganglia homogenates in these experiments. In addition, an experiment was performed with AP incubated in hemolymph, the primary extracellular fluid in *Aplysia*. In this experiment, no Leu–Leu processing was observed.

After the AP solution was added to the abdominal ganglia homogenates, the intensity of the AP and AP S[1–20]K peaks increased, but no further processing occurred. Leu–Leu processing products were not detected during the 15 min run. Interestingly, AP S[1–20]K was also observed in the spectra acquired from the buccal ganglia incubation, but at a much lower intensity than most of the Leu–Leu products.

Unlike the spatial differentiation of Leu–Leu processing observed with AP, cleavage at Leu–Leu sites in porcine angiotensinogen occurred in an equivalent manner in both ganglion homogenates. Angiotensinogen has 14 residues and only one Leu–Leu site. With a single Leu–Leu site, only three possible Leu–Leu cleavage products can be produced with masses that are easily differentiated with MALDI. Of the three possibilities, two of the products were observed with incubations in the abdominal and buccal ganglia homogenates (Fig. 3). In all spectra, the uncleaved angiotensinogen peak was dominant. The two Leu–Leu product peaks were detected at roughly one third the intensity of the angiotensinogen peak at the beginning of the time course. Both peaks decreased in intensity throughout the experiment and were undetectable by the end of 15 min, likely indicating that the peptides continued to be processed, possibly into forms below 500 Da, the lower mass limit of MALDI spectra acquired in this experiment.

Leu–Leu processing of human secretin was also observed in a differential manner in the *Aplysia* CNS (Fig. 4). When the peptide solution was incubated with buccal ganglia homogenate supernatant, some Leu–Leu processing products were detected. Similar to the angiotensinogen incubation, the peaks were much less intense than the intact peptide peak. Multiple processing products were produced consistently, some of which were attributed to Leu–Leu cleavages, also in a time-dependent manner. There was no clear time/size correlation as in the AP/buccal ganglia experiment (larger peptides produced first and consumed into smaller products); some peaks grew and disappeared during the 15 min run. The results of the secretin incubation in the abdominal ganglia homogenate supernatant were less straightforward, likely due to the low intensity that makes detection difficult. The peaks at 798 and 1784 Da corresponded with peaks observed in the buccal ganglia, however, the peak at 2531 Da was novel in this study. Also, the peaks were present at lower intensities than the products in the buccal ganglia.

Similar incubations were performed with the HPLC fractions from the buccal ganglia homogenates. AP was added to all fractions and then monitored with MALDI MS to determine if cleavage at Leu–Leu sites occurred. Two of the fractions, fractions 4 and 6, contained Leu–Leu cleavage products following incubation. One of the fractions, fraction 4, was then subjected to pH changes and the incubations repeated. At an acidic pH of 5.5, the Leu–Leu endoprotease was active, producing intense Leu–Leu products (Fig. 5). Similar products were observed when the pH was raised to 7.2, although the MS peaks were present at slightly smaller intensity. When the pH was raised again to 9.1, however, a noticeable difference in the generation of Leu–Leu products was observed. Few Leu–Leu products were observed

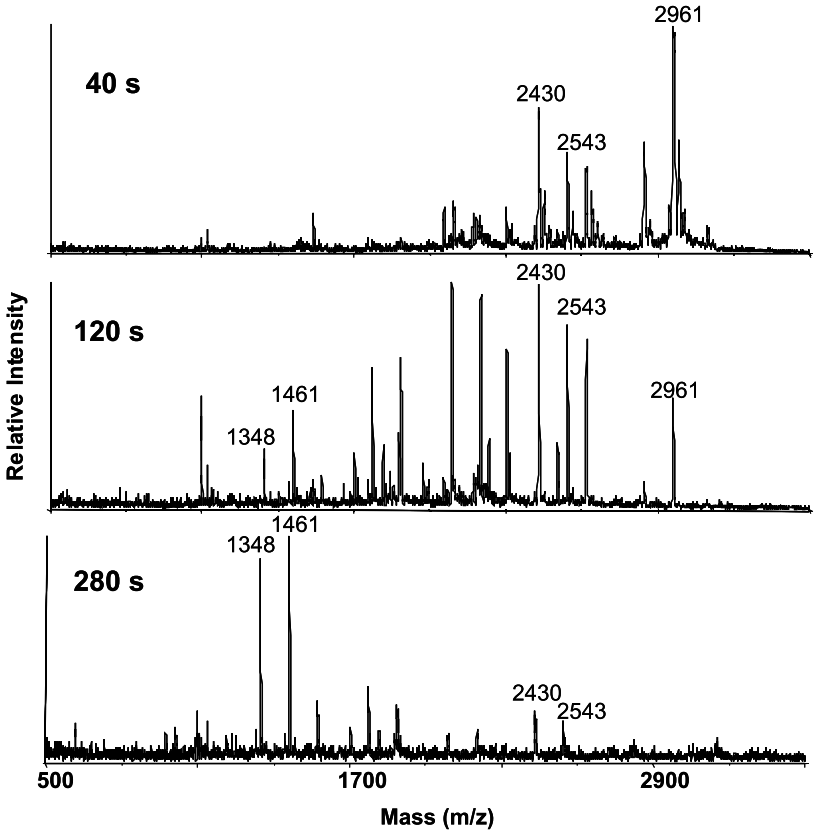

Fig. 2 Time course of AP processing. Mass spectra of the processing of acidic peptide in supernatant of buccal ganglia homogenate at room temperature at various lengths of time after the addition of AP. During the experiment, larger processing products are consumed and smaller products are produced. The peptides correspond to the following masses: **LL**TSNKDEEQRE**LL**KAISN**LL**D, $[M + H]^+ = 2543$ Da; **L**TSNKDEEQRE**LL**KAISN**LL**D, $[M + H]^+ = 2430$ Da; **LL**TSNKDEEQRE or **L**TSNKDEEQRE**L** or TSNKDEEQRE**LL**, $[M + H]^+ = 1461$ Da; and **L**TSNKDEEQRE or TSNKDEEQRE**L**, $[M + H]^+ = 1348$ Da.

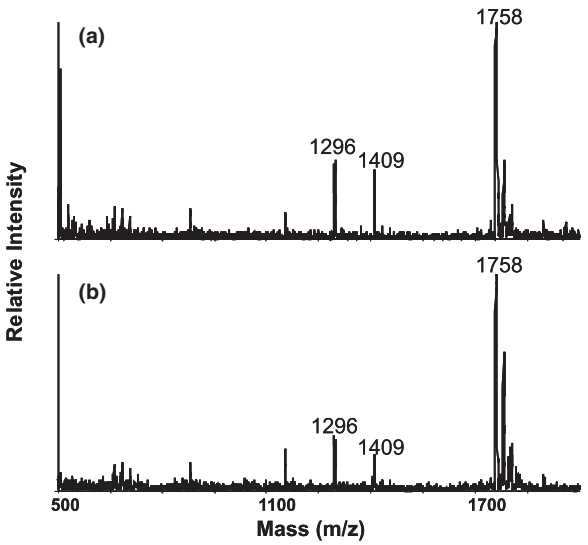

Fig. 3 Mass spectra of incubations of porcine angiotensinogen 1–14 (DRVYIHPFH**LL**VYS, $[M + H]^+ = 1758$ Da) in the supernatant of abdominal (a) and buccal (b) ganglia homogenates. Two Leu–Leu products are observed, DRVYIHPFH**L**, $[M + H]^+ = 1296$ Da and DRVYIHPFH**LL**, $[M + H]^+ = 1409$ Da.

and with considerably lower MS peak intensity, perhaps suggesting a weakening of the endoprotease-peptide bond at higher hydroxide concentrations.

The same HPLC fraction used in the pH study was also treated with EDTA to determine if the endoprotease responsible for Leu–Leu cleavage was a metalloenzyme. The fraction was exposed to an excess of EDTA in a specially constructed vial containing a dialysis membrane. One portion of the fraction was incubated with a Leu–Leu containing peptide and monitored by MALDI MS, although no Leu–Leu processing products were detected. Another portion was reacted with tap water to provide a supply of metal ions for the enzyme center. With the addition of metal ions, the Leu–Leu processing products were detected.

Discussion

The processing of prohormones is a sequence-specific and tissue-specific phenomenon. The elucidation of new enzymatic processes is important for the understanding of prohormone processing and for finding new processing enzymes. In this study, we investigated a fairly novel neuropeptide processing event, cleavage at Leu–Leu residues. The process was temporally and spatially characterized with different peptide sequences within the *Aplysia* CNS. While some variations were observed in the processing of the peptides, all were cleaved at their Leu–Leu residues. The process does not appear to occur at a specific bond; cleavages

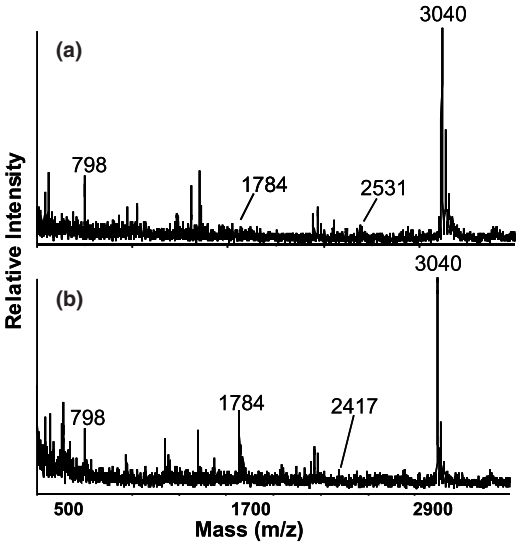

Fig. 4 Mass spectra of incubations of human secretin (HSDGTFTSELSRLREGARLQR**LL**QGLV-NH2, $[M + H]^+ = 3040$ Da) in the supernatant of abdominal (a) and buccal (b) ganglia homogenates. In the abdominal ganglia homogenate, three Leu–Leu products are observed, RLQR**LL**, $[M + H]^+ = 798$ Da, SELSRLREGARLQR**L**, $[M + H]^+ = 1784$ Da, and HSDGTFTSELSRLREGARLQR**L**, $[M + H]^+ = 2530$ Da. In the buccal ganglia homogenate, three peptides are produced by cleavage at Leu–Leu sites. Two of the peptides are identical to those seen in the abdominal ganglia, the 798 and 1784 Da products. A new peptide, HSDGTFTSELSRLREGARLQR, $[M + H]^+ = 2417$ Da, is also observed.

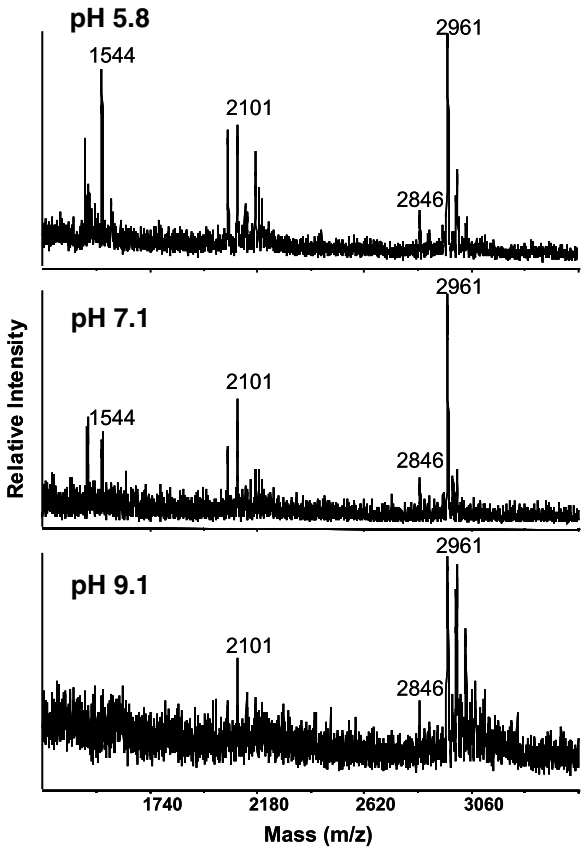

Fig. 5 Mass spectra of incubations of *Aplysia* acidic peptide (AP) with an HPLC fraction of pooled buccal ganglia at different pHs. The peptides produced by cleavage at Leu–Leu sites are EEQRE**LL**KAISN**L**, $[M + H]^+ = 1544$ Da; SNKDEEQRE**LL**KAISN**LL**, $[M + H]^+ = 2101$ Da; and SSGVS**LL**TSNKDEEQRE**LL**KAISN**LL**, $[M + H]^+ = 2846$ Da. At acidic and neutral pH, there are multiple Leu–Leu products produced. At basic pH, the production of Leu–Leu products decreases significantly.

were observed both adjacent (N- and C-terminus) to and between the two Leu residues.

With most of the Leu–Leu studies, the results reported are from incubations performed with the supernatant of ganglia homogenates. However, the initial experiments involved the incubation of AP with complete homogenates of the buccal ganglia. The processing observed in these experiments was identical to the processing later seen when AP was incubated with the buccal homogenate supernatant. All subsequent experiments were performed with ganglia homogenate supernatants and the results reported in this paper exclusively involve incubations with supernatants. This procedure was used for two reasons: to minimize MS signal from the membrane lipids and to confirm whether the enzyme responsible for Leu–Leu cleavage was cytoplasmic or membrane bound.

Compared to basic cleavages at Lys and Arg, the hydrophobic Leu–Leu cleavage requires a different protease. An interesting question involves the specificity of the Leu–Leu cleavages. Is there something specific about this particular amino acid combination or would a similar cleavage pattern be observed for any pair of hydrophobic amino acids? Previous work has noted cleavages between Leu–Ser and Ala–Ala sites (Suchanek *et al*. 1978; Hudson *et al*. 1981). In addition, a mammalian serine protease of the subtilisin/kexin type that cleaves at threonine-serine residues has been identified (Seidah *et al*.; 1999). In *Aplysia* prohormones, we have observed cleavage at other hydrophobic pairs: proELH (Ser–Leu and Leu–Thr), proL5-67 (Leu–Phe), proInsulin (Ile–Leu), and pro A-ELH (Val–Thr) (Garden *et al*. 1998; Li *et al*. 1998; Floyd *et al*. 1999; Li *et al*. 1999). Future work will focus on characterizing the hydrophobic pairs, Ile–Ile, Leu–Ile, and Ile–Leu, as well as additional combinations with Phe, Val, and Ala. Some of these pairs are observed in *Aplysia* prohormones; for example, Phe–Val, Phe–Phe, and Phe–Ala, are commonly found near the C-terminus of neuropeptides. While cleavages between these residues have not been noted as yet, Leu–Leu residues are also present in the prohormones that are not utilized. Therefore, it may be that, like the basic cleavage sites, these hydrophobic pairs may be cleaved only a percentage of the time in specific cells.

Can the observed processing be caused by a known enzyme that processes at Leu sites, for example renin or chymotrypsin, or is this pattern indicative of a novel protease? To our knowledge, neither renin nor angiotensinogen have been identified in *Aplysia,* although angiotensinogen-like immunoreactivity has been demonstrated in portions of the *Aplysia* CNS (Gonzalez *et al*. 1995). Also, cleavage by renin in other species is highly substrate specific, only occurring with angiotensinogen and cleaving between the two Leu residues. As we observe cleavages both between and adjacent to the Leu–Leu site, it appears that the protease is significantly different than the known renins. A previous study described the cleavage of human, rat, and porcine proinsulin C-peptides at Leu–Ala bonds and attributed the process to an unidentified enzyme with chymotrypsin-like specificity. A similar pattern was also observed with prorelaxin, a homologue of proinsulin, at a Leu-Ser bond and credited to a chymotrypsin-like enzyme (Steiner *et al*. 1984). With chymotrypsin, cleavage is observed uniquely at the C-terminus side of a Leu residue. As we observe cleavage at the N- as well as the C-terminus side, the process has unique specificity. Thus far, chymotrypsin has not been isolated in *Aplysia*, though it has been identified in other invertebrates (Polanowski and Wilusz 1996; Chopin *et al*. 2000). We believe that the pattern of processing observed with these experiments is explained by uncharacterized protease(s). Based on our results with intracellular (ganglia homogenate supernatants) and extracellular (hemolymph) fluids, we believe the enzyme responsible is a water-soluble, intracellular protein. In addition, the protease appears to be most active in acidic or neutral pH ranges, consistent with an endoprotease in the secretory pathway. The inhibition by EDTA also indicates that the enzyme is a metalloprotease or a protease that requires a metal for activity. Further studies will focus on identifying this endopeptidase.

As noted by Veenstra, ambiguous processing sites (ones that may or may not be cleaved) may exist to allow differential cleavage of the same precursor by different convertases or cleavage by proteases after release of the peptides (Veenstra 2000). This variance allows for a greater diversity of products from fewer starting materials. However, while this pluralism of specificity allows for more biological diversity and function from fewer starting materials, it makes the task of predicting peptide cleavages more challenging. Only by profiling additional consensus cleavage sites and gaining a more accurate profile of the unique specificities of the individual enzymes can improved predictions for the processing of new prohormones be made.

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